Manuscript title: Genetic variability of the *ABCC2* gene and clinical outcomes in pancreatic cancer patients

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) has an extremely poor prognosis, caused by various factors, such as the aggressiveness of the disease, the limited therapeutic options and the lack of early detection and risk markers. The ATP binding cassette subfamily C member 2 (ABCC2) protein plays a critical role in response to various drugs and is differentially expressed in gemcitabine sensitive and resistant cells. Moreover, Single Nucleotide Polymorphisms (SNPs) in the gene have been associated with differential outcomes and prognosis in several tumour types. The aim of this study was to investigate the possible association between SNPs in the ABCC2 gene and overall survival in PDAC patients. We analysed 12 polymorphisms, including tagging-SNPs covering all the genetic variability of the ABCC2 gene, and genotyped them in 1415 PDAC patients collected within the PANcreatic Disease ReseArch (PANDoRA) consortium. We tested the association between ABCC2 SNPs and PDAC overall survival (OS) using Cox proportional hazard models. We analysed PDAC patients dividing them by stage and observed that the minor alleles of three SNPs showed an association with worse OS (rs3740067: HR=3.29, 95% CI 1.56-6.97, p=0.002, rs3740073: HR=3.11, 95% CI 1.52-6.38, p=0.002 and rs717620: HR=2.90, 95% CI 1.41-5.95, p=0.004 respectively) in stage I patients. In patients with more advanced PDAC we did not observe any statistically significant association. Our results suggest that rs3740067, rs3740073, and rs717620 could be promising prognostic markers in stage I PDAC patients.

Summary

We investigated the possible association between SNPs in the *ABCC2* gene and overall survival in pancreatic ductal adenocarcinoma patients and observed that the minor alleles of three SNPs showed an association with worse OS in stage I patients.

Abbreviations

PDAC - pancreatic ductal adenocarcinoma

ABCC2 - ATP-Binding Cassette subfamily C member 2

SNP - single nucleotide polymorphisms

GWAS - genome-wide association studies

PANDoRA - PANcreatic Disease ReseArch

OS - overall survival

MAF - minor allele frequency

HWE - Hardy–Weinberg equilibrium

HR - hazard ratio

DNMBP - Dynamin Binding Protein

eQTL - expression quantitative trait loci

CI - confidence interval

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease with an annual incidence of 12.2 cases/100000 subjects and is the fourth most common cause of death from cancer in the European Union (1). PDAC showed an increasing incidence and mortality over the last several years (2). This unfavourable prognosis is mostly caused by the aggressiveness of the disease and the absence of specific symptoms, which make early diagnosis difficult. Indeed, more than half of PDAC patients have distant metastases at the time of diagnosis (3). Five-year survival rate for PDAC patients is 5–7% and one-year survival is achieved in less than 20% of the cases (4).

Many resectable patients undergo neo-adjuvant and/or adjuvant treatment, while palliative chemotherapy remains the only option for almost all patients with metastatic disease (5,6). Gemcitabine is often employed as first-line chemotherapy regimens in these advanced cases, either as a single agent or in combination with Nab-paclitaxel (5). Advances in therapy using new drugs and combined drugs have only achieved incremental improvements in overall survival by around two months. Several studies suggest differential effectiveness in subsets of patients, so that the current practice may increase toxicity without increasing efficacy for many patients, and can provide notable benefit for undefined subgroups of patients (5–7). As a consequence, there is an urgent need for better understanding the pharmacogenetics of PDAC in order to improve patient selection for current treatment options. Several studies focused on the role of ATP-binding cassette (ABC) genes in pancreatic cancer chemoresistance, considering both their expression or their genetic variability (8–12).

In particular, a study reporting on the differential gene expression between cells sensitive and resistant to gemcitabine showed the differential expression of the ATP-Binding Cassette subfamily C member 2 (*ABCC2*) gene (13). ABCC2, also known as multi-drug resistance protein 2 (*MRP2*), plays a role in detoxification and chemoprotection, transporting several xenobiotic compounds outside the cell and modulating the pharmacokinetics of many drugs (14). Several studies showed that single nucleotide polymorphisms (SNPs) of the *ABCC2* gene are associated with altered distribution, metabolism, and elimination of a plethora of drugs (15–19). The possible influence of the genetic background in survival of PDAC patients has been suggested by a number of studies including five genome-wide association studies (GWAS) (20–28). Considering that ABCC2 may be involved in the process of response to therapy, in this study we tested the possible association between the genetic variants in the *ABCC2* gene and the survival of PDAC patients.

Materials and methods

Study population

This study was conducted within the PANcreatic Disease ReseArch (PANDoRA) consortium, described in detail elsewhere (29). PDAC patients (n=1415) were collected in 7 European countries (Italy, Germany, Hungary, Czech Republic, Poland, Lithuania, United Kingdom). For each patient, information about the country of origin, cancer staging (according to American Joint Committee on Cancer TNM system 7th version), gender and age at diagnosis was collected. Furthermore, detailed information concerning overall survival (OS) was recorded as well.

The characteristics of patients are described in **Table I.**

Ethics statement

All subjects signed a written consent form. Ethical approval for the PANDoRA study protocol was received from the Ethics Commission of the Medical Faculty of the University of Heidelberg (S-565/2015).

Selection of genes and polymorphisms

We utilised a tagging SNP approach. The entire set of common genetic variants, with minor allele frequency (MAF) ≥5% in Caucasians was downloaded from the International HapMap Project (30). Tagging SNPs were selected using the Tagger algorithm available through Haploview (31), using pairwise SNP selection with a minimum r^2 threshold of 0.8. This process resulted in a selection of 12 tagging SNPs for *ABCC2*, with a mean r^2 of the selected SNP with the SNPs they tag of 0.981. This selection, therefore, captures a very high degree (over 95%) of the known common variability in *ABCC2*. The 12 SNPs selected are shown in **Table II**.

DNA extraction and genotyping

Blood was collected using standard EDTA collection tubes and stored at -20C° until DNA isolation. DNA was extracted from whole blood using the Qiagen mini kit (Qiagen, Hilden, Germany) or the All-Prep Isolation kit (Qiagen) according to the manufacturer's protocol. Both kits are standard spin column

extraction methods. For better standardization of the procedure the DNA was isolated using the QIAcube instrument (Qiagen, Hilden, Germany). DNA concentration was checked with a spectrophotometer and purity was assessed with the ratio of absorbance at 260nm and 280nm. DNA was then stored at -20C° until use at the German Cancer Research Center in Heidelberg. Genotyping was performed using the allele-specific KASP genotyping system (KBiosciences, Hoddesdon, UK) as recommended by the manufacturer which is a competitive PCR based on fluorescent resonance energy transfer (FRET) principle. Each 384-well plate was prepared to contain a minimum of 8 (2%) negative controls (i.e. wells in which every reagent was used except for the DNA template) and around 9% of the samples were duplicated, for quality control. The order of DNA samples of cases and controls was randomised on the plates. PCR plates were read on a ViiA7 real-time instrument (Applied Biosystems, Foster City, California, United States) and FLUOstar® Omega sequence detection system (BMG LABTECH Ortenberg, Germany). The ViiA7 RUO Software, version 1.2.2 (Applied Biosystems) and the KlusterCaller software (LGC, Teddington, UK), were used to determine genotypes.

Statistical analysis

The observed genotype frequencies of all SNPs in PDAC cases were tested for deviation from Hardy–Weinberg equilibrium (HWE) using Pearson's chi-square test. Overall survival (OS) was defined as the time interval between PDAC diagnosis and death or the last date when the patient was still alive. Association with OS was calculated as hazard ratio (HR) using Cox regression multivariate analysis adjusted for age, gender, country of origin and stage of PDAC. Association estimates were calculated according to dominant and co-dominant models of inheritance with the major allele as a reference. We also performed a stratified analysis by stage to evaluate the associations between SNPs and OS in different PDAC progression stages.

In order to take into account multiple comparisons, we calculated the effective number of independent genetic markers (N=8) using the (M_{eff}) method by the spectral decomposition (SpD) of matrices of pairwise LD between SNPs (32,33). The critical value for statistical significance was corrected by the Bonferroni method to a significance threshold of p=0.006 (0.05/8).

Bioinformatic analysis

We used several bioinformatic tools to assess the possible functional relevance for the SNP showing the most significant association with OS. RegulomeDB (http://regulome. stanford.edu/) and HaploReg v4.1

(http://archive.broadinstitute.org/mammals/haploreg/haploreg.php) was used to identify the regulatory potential of the region nearby each SNP (34,35). The GTEx portal web site (http://www.gtexportal.org) was used to identify potential associations between the SNPs and expression levels of nearby genes (eQTL) (36). Additionally, we used the SNAP software (http://archive.broadinstitute.org/mpg/snap/) to find SNPs in LD with the SNP that showed the strongest association with OS using a threshold of $r^2 = 0.70$ (37).

Results

All the selected SNPs were in HWE (p>0.05) in our study population. The genotyping concordance between duplicate samples (N=130) exceeded 99% and the average SNP call rate was 96% (92%–99%).

We did not observe any statistically significant association when considering all patients together (**Supplementary table I)**. We repeated the analyses stratifying by stage, given that it strongly influences survival. Three polymorphisms (rs3740067, rs3740073, rs717620) were found to have a statistically significant association (P<0.006) with PDAC survival in patients in stage I. Specifically, we observed an association with shorter OS for the G allele of the rs3740067 SNP (HR_{dom}=3.29, 95% CI 1.56-6.97, p=0.002), the T allele of the rs3740073 SNP (HR_{dom}=3.11, 95% CI 1.52-6.38, p=0.002) and the T allele of the rs717620 SNP (HR_{dom}=2.90, 95% CI 1.41-5.95, p=0.004) (**Table III**). **Figure 1** shows the Kaplan-Meier curves for the association between *ABCC2* SNPs-(rs3740067, rs3740073, rs717620) and OS for patients in stage I. Kaplan-Meier curves for all patients are shown in **supplementary figure 1**. Additionally, we analyzed jointly patients in stage I and II, but we did not observe any statistically significant association (data not shown).

We analysed the SNPs showing the most significant associations using bioinformatic tools to predict the possible biological functions. The three significant SNPs showed different scores in RegulomeDB. Specifically, rs3740067 has a score of 3a, indicating the possible presence of a transcription factor binding motif, a DNase sensitivity peak, and any protein motif alteration, a score of 6 for rs3740073 indicates the absence of annotations and 4 for rs717620 indicates the possible presence of a transcription factor binding motif and a DNase sensitivity peak.

GTEx (36) shows that rs3740067, rs3740073, and rs717620 alter the expression of four genes: *ABCC2*, Dynamin Binding Protein (*DNMBP*), DNMBP Antisense RNA 1 (*DNMBP-AS1*) and CWF19 Like 1 (*CWF19L1*) in eighteen different tissues (**Supplementary table II**).

Discussion

PDAC shows a mortality rate that is very similar to the incidence and it is estimated that by 2030 it will be the second cancer for mortality (38). A possible way to improve treatment efficacy could be to consider the genetic background of the patients and possibly tailor a personalized treatment. There is convincing evidence pointing to a possible role of ABCs genes in PDAC drug resistance. For instance, Konig and colleagues examined the expression level of nine ABC genes in normal and tumoral pancreatic tissues, finding the majority of them to be expressed. Schaarschmidt reported the expression of *ABCB1* and *ABCC2* genes in both normal and inflamed pancreatic tissue (9,12). In a recent paper Huang and colleagues proposed a possible association between ABCC2 expression and gemcitabine sensitivity (8). Genetic variants in transporter pumps could influence the quantity of drugs that reaches the target and the time that the drug remains in the cell to exert its effect. For example, Pang and colleagues found that a particular haplotype in the ABCB1 gene was associated with increased gemcitabine sensitivity (11). Tanaka and collaborators observed several associations between ABC gene variants and PDAC outcome (10). In our study we focused on the polymorphisms of ABCC2, an ATP dependent efflux pump that has been investigated in many pharmacogenetics studies given its central role in drug transport and excretion.

Considering all 1415 PDAC cases together we observed no statistically significant association between the 12 selected SNPs and disease outcome, however when stratifying for stage we identified three polymorphisms showing an association with OS in stage I patients. In particular, we observed statistically significant associations between rs3740067 (p=0.002), rs3740073 (p=0.002) and rs717620 (p=0.004) and shorter survival. The rs3740067 and rs3740073 polymorphisms have already been studied in the context of studies on risk of various cancers and survival, but did not show significant associations (39,40). Tanaka et al (10) performed an investigation on ABCs genes SNPs on 154 ethnically diverse pancreatic cancer patients. Two *ABCC2* SNPs were included in that study. The major allele of rs2273697 was associated with decreased survival and with worse response to therapy. We have used SNP rs11190291, which is in perfect LD (r2=1) with rs2273697. In our data rs11190291 does not show a statistical significance overall, but the major allele is consistently associated with worse survival, and when analyzing only the stage IV patients it reaches a nominal significance (p=0.015). The other SNP analyzed by Tanaka and colleagues, rs3740066, is in perfect LD with our SNP rs3740067. Also for these SNPs the association goes in the same direction (minor allele of both SNPs associated with worse survival) although in our data the association is significant if considering stage I patients, but not in the overall population. In the report by Tanaka and colleagues this SNP does not show any significant association. Overall, the direction of associations seems to be consistent between the two reports, however the rather small numbers used by Tanaka did not allow them to

perform stratified analysis and therefore the two results are not directly comparable. On the other hand, despite it has never been studied in relation to PDAC patient survival, *ABCC2*-rs717620 is a potentially functional SNP situated in the promoter region of the gene. In a relatively large study on lung cancer survival the T allele of the rs717620 has been associated with shorter progression-free survival (19). Furthermore the minor allele (T) was associated with increased risk of neurological toxicity in cancer patients treated with the 5-fluorouracil, and with increased platinum-related toxicity in lung cancer patients (41,42). Increased sensitivity to the toxic effects of chemotherapeutic agents can lead to premature discontinuation of therapy, reducing the patient's chances of survival. All these reports are in agreement with the results emerging from the present study, suggesting a worse prognosis for carriers of the T allele of *ABCC2*-rs717620. It is noteworthy that the polymorphism seems to have a stronger effect on the first stages of the disease, since in lung cancer it is associated with progression-free survival and in our study with a differential survival in stage I patients. Considering the function of the protein, i.e. exporting xenobiotics from the cells, it is plausible that an altered expression could be associated with a decreased drug efficacy and/or with an increase in toxicity. Using several bioinformatic tools such as GTEx we tested in silico this hypothesis, however the tools did not provide straightforward results. For example the regulome DB score was 4, indicating a possible presence of a transcription factor binding motif or a DNase sensitivity peak, however GTEx did not identify any eQTLs for *ABCC2*-rs717620 in the pancreas.

According to GTEx the three alleles associated with shorter survival are associated with the increased expression of *DNMBP* in several tissues, but not in the pancreas. *DNMBP* is a gene that codes for a scaffold protein for dynamines that are involved in scission of newly formed vesicles from the membranes. Functional studies suggest that dynamin proteins can participate in promoting cell migration and metastasis in PDAC cellular and mouse models (43,44). In addition, in a recent study, dynamins and their complexes have been hypothesized as important therapeutic targets in cancer (45). The association between aggressiveness of cancers and expression of dynamins and their protein complexes is well matched with our data, where the alleles associated with decreased survival are associated with an increase in *DNMBP* gene expression, as shown in GTEx. In light of the fact that the GTEx associations between SNPs and gene expression are not observed in the pancreatic tissue, these results need to be taken with caution. However it is potentially interesting to note that in all the tissues present in GTEx the associations between alleles and gene expression seems to be consistent (i.e. the minor alleles are always associated with increased expression). Based on these observations we can hypothesize that the association is the same in the pancreas.

One of the major strengths of this study is its size, since with a total of 1415 subjects it is one of the largest on pancreatic cancer survival. Additionally, our selection of SNPs provides a wide coverage of genetic variability in the *ABCC2* gene region.

A possible limitation of our study is not having the information regarding the therapies to which the patients were subjected. This type of data could have producted greater value to the associations and their possible biological functions. In addition, the associations that we find significant have been identified in a small subgroup of patients. The vast majority of the patients enrolled in this study (83%) received surgery and therefore it is not possible to generalize our findings. Moreover, all study subjects were Europeans and it is not possible to generalize our findings to other ethnic groups.

Conclusion

In conclusion, our study showed three promising polymorphisms associated with overall survival in stage I pancreatic cancer patients. The polymorphic variant of the promoter (rs717620) has been already found to be associated with survival in cancer patients, supporting our findings, even though a biological explanation remains elusive. Finding an association in the early stage of the cancer is potentially interesting because treatment could have a greater impact on the disease outcome.

Author roles

D.C. conceived the study. M.G., A.A.G. and P.P.G. performed experimental work. D.C. and M.G. performed data analysis. All other authors contributed to the collection of samples and data. M.G., D.C., and F.C. drafted the manuscript, and all other authors took part in its critical revision.

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Table I. Information about the patients from PANDoRA consortium analysed in our study.

	PDAC cases
Country/region	
Germany	410
Northern Italy	343
Central Italy	80
Southern Italy	43
Hungary	195
Czech Republic	146
United Kingdom	73
Poland	72
Lithuania	53
Total	1415
Sex	
Male	57.4%
Female	42.6%
Median age	65.5
(25^{th} – 75^{th} percentile)	58.5-72.0
Median OS in months	11.77
Stage (median OS in months) [percentage of patients operated]	
I	88 (15.50) [97%]
II	748 (14.50) [98%]
III	145 (10.55) [86%]
IV	434 (8.51) [74%]

Table II. Annotation of the *ABCC2* gene selected SNPs

SNP	Position on chr 10 [A]	Alleles	MAF [B]	Consequence
rs717620	99782821	C>T	T=20%	Non Coding Transcript Variant
rs7393105	99787264	C>A	C=44%	Intron Variant
rs4148388	99790008	G>A	G=41%	Intron Variant
rs2756109	99798989	G>T	G=43%	Intron Variant
rs11190291	99806253	C>T	T=20%	Intron Variant
rs3740073	99817203	T>C	T=39%	Intron Variant
rs4077146	99829571	G>A	A=25%	Intron Variant
rs7476245	99834972	G>A	A=5%	Intron Variant
rs3740067	99844024	C>G	G=37%	Intron Variant
rs3740065	99845936	A>G	G=12%	Intron Variant
rs8187710	99851537	G>A	A=7%	Missense Variant (Cys>Tyr)
rs11190297	99858346	G>T	T=6%	None

[A] Genome Reference Consortium Human Build 38 patch release 7 (GRCh38.p7)

[B] Minor Allele Frequency; 1000Genomes_EUR

Table III. Cox analysis stratified by stage of the three polymorphisms significantly associated (P<0.006) with OS.

SNP	Genotype	Stage I				Stage II				Stage III				Stage IV			
		N	HR	95% CI	P	N	HR	95% CI	P	N	HR	95% CI	P	N	HR	95% CI	P
rs3740067	CC	29	1	-	-	293	1	-	-	69	1	-	-	149	1	-	-
	CG	39	3.07	(1.41-6.68)	**0.0046**	340	0.99	(0.82-1.19)	0.8829	57	0.86	(0.55-1.35)	0.5248	193	1.17	(0.92-1.49)	0.1899
	GG	12	4.16	(1.6-10.86)	**0.0035**	102	0.98	(0.75-1.27)	0.8535	15	0.76	(0.39-1.5)	0.4314	55	1.22	(0.86-1.72)	0.2686
	dom		3.29	(1.56-6.97)	**0.0018**		0.98	(0.83-1.17)	0.8532		0.84	(0.55-1.28)	0.414		1.18	(0.94-1.48)	0.1475
	Total	80				735				141				397			
rs3740073	CC	29	1	-	-	262	1	-	-	58	1	-	-	144	1	-	-
	CT	43	3.11	(1.46-6.61)	**0.0032**	339	0.93	(0.77-1.12)	0.4337	57	1.32	(0.84-2.05)	0.2259	178	1.24	(0.98-1.58)	0.0727
	TT	13	3.12	(1.26-7.7)	0.0137	105	1.12	(0.86-1.47)	0.3908	15	0.72	(0.33-1.54)	0.3956	58	1.2	(0.85-1.69)	0.295
	dom		3.11	(1.52-6.38)	**0.0019**		0.97	(0.81-1.16)	0.7143		1.16	(0.76-1.79)	0.4839		1.23	(0.98-1.54)	0.0685
	Total	85				706				130				380			
rs717620	CC	50	1	-	-	429	1	-	-	89	1	-	-	230	1	-	-
	CT	24	2.8	(1.34-5.86)	0.0061	199	1.09	(0.9-1.34)	0.3747	40	0.78	(0.47-1.31)	0.3521	135	1.12	(0.88-1.43)	0.3473
	TT	2	5.78	(0.36-93.9)	0.2171	26	0.73	(0.45-1.18)	0.2023	2	4.35	(0.99-19.18)	0.052	12	0.99	(0.53-1.85)	0.9772
	dom		2.9	(1.41-5.95)	**0.0038**		1.04	(0.86-1.26)	0.6967		0.84	(0.51-1.38)	0.4885		1.11	(0.88-1.4)	0.3843
	Total	76				654				131				377			

HR: hazard ratio; CI: confidence interval; P: p-value; dom: dominant model.

Figure legends

Figure 1. Kaplan Meier survival analysis.
Kaplan Meier survival curves of three polymorphisms (**A**) rs3740067, (**B**) rs3740073, (**C**) rs717620 were found to have a statistically significant association with PDAC survival in patients in stage I.